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Exploring the Contribution of Candidate Genes to Artemisinin Resistance in *Plasmodium falciparum*[▽]

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The reduced *in vivo* sensitivity of *Plasmodium falciparum* has recently been confirmed in western Cambodia. Identifying molecular markers for artemisinin resistance is essential for monitoring the spread of the resistant phenotype and identifying the mechanisms of resistance. Four candidate genes, including the *P. falciparum mdr1* (*pfmdr1*) gene, the *P. falciparum ATPase6* (*pfATPase6*) gene, the 6-kb mitochondrial genome, and *ubp-1*, encoding a deubiquitinating enzyme, of artemisinin-resistant *P. falciparum* strains from western Cambodia were examined and compared to those of sensitive strains from northwestern Thailand, where the artemisinins are still very effective. The artemisinin-resistant phenotype did not correlate with *pfmdr1* amplification or mutations (full-length sequencing), mutations in *pfATPase6* (full-length sequencing) or the 6-kb mitochondrial genome (full-length sequencing), or *ubp-1* mutations at positions 739 and 770. The *P. falciparum* CRT K76T mutation was present in all isolates from both study sites. The *pfmdr1* copy numbers in western Cambodia were significantly lower in parasite samples obtained in 2007 than in those obtained in 2005, coinciding with a local change in drug policy replacing artesunate-mefloquine with dihydroartemisinin-piperaquine. Artemisinin resistance in western Cambodia is not linked to candidate genes, as was suggested by earlier studies.

Antimalarial drug resistance is the single most important threat to global malaria control. Over the past 40 years, as first-line treatments (chloroquine and sulfadoxine-pyrimethamine) failed, the malaria-attributable mortality rate rose, contributing to a resurgence of malaria in tropical countries (11). In the last decade, artemisinins, deployed as artemisinin combination therapies (ACTs), have become the cornerstone of the treatment of uncomplicated falciparum malaria (20) and, in conjunction with other control measures, have contributed to a remarkable decrease in malaria morbidity and mortality in many African and Asian countries (4). The recent confirmation of the reduced artemisinin sensitivity of *Plasmodium falciparum* parasites in western Cambodia has therefore alarmed the malaria community (6). A large containment effort has been launched by the World Health Organization, in collaboration with the national malaria control programs of Cambodia and neighboring Thailand. The resistant phenotype has not been well characterized and is not well reflected by the results of conventional *in vitro* drug susceptibility assays. No molecular marker has been identified, which impedes surveillance studies to monitor the spread of the resistant phenotype. Identification of molecular markers would give insight into the mechanisms underlying artemisinin resistance and the mechanism of antimalarial action of the artemisinins.

Mutations in several candidate genes have been postulated to confer artemisinin resistance. (i) *P. falciparum mdr1* (*pfmdr1*) encodes the P-glycoprotein homologue 1 (Pgh1), which belongs to the ATP-binding cassette transporter superfamily, members of which couple ATP hydrolysis to the translocation of a diverse range of drugs and other solutes across the food vacuole and plasma membranes of the parasite (Fig. 1) (5). The gene is located on chromosome 7, is 4.2 kb in length, and contains only one exon. Mutations in and, more importantly, amplification of the wild-type gene confer resistance to the 4-methanolquinoline mefloquine, presumably through an increased ability to efflux the drug (15, 16). Mutations and amplification of the gene have also been associated with reduced *in vitro* susceptibility to the artemisinins (7, 16). *In vivo* selection of the *pfmdr1* 86N allele after artemether-lumefantrine treatment has been observed in Africa (17).

(ii) *P. falciparum ATPase6* (*pfATPase6*) encodes the calcium-dependent sarcoplasmic/endoplasmic reticulum calcium ATPase, which was shown to be a target for the artemisinin drugs in *Xenopus* oocytes (8). The gene is 4.3 kb in length and has three exons on chromosome 1. A single amino acid change

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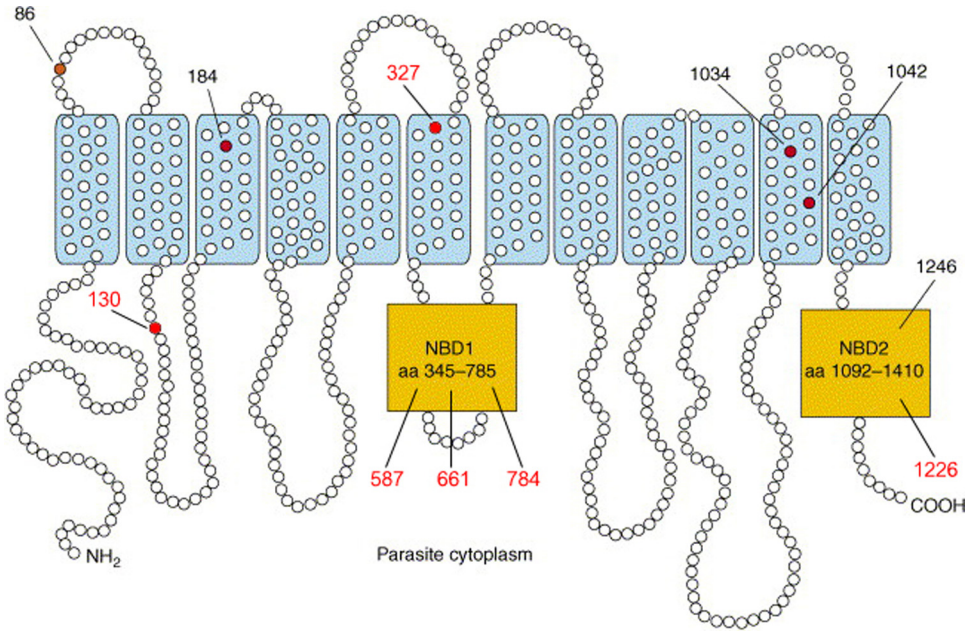

FIG. 1. Predicted structure and representative haplotypes of *P. falciparum* multidrug resistance transporter. PfMDR1 is predicted to have 12 transmembrane domains, with its N and C termini located on the cytoplasmic side of the digestive vacuole membrane (adapted from reference 19). Mutations identified in *pfmdr1* full-length sequences from Pailin and WangPha are indicated by the red circles. aa, amino acid.

in *pfATPase6*, L263E, is associated with resistance to artemisinins in this model (8, 18). Mutation S769N in *pfATPase6* in *P. falciparum* isolates from French Guiana was associated with decreased *in vitro* sensitivity to artemether (10). However, it is unclear whether mutations in *pfATPase6* are associated with artemisinin resistance *in vivo* (1).

(iii) The electron transport chain in the mitochondrial inner membrane is key to the malaria parasite's capacity to produce ATP. Since activation of the endoperoxide bridge in the artemisinins by an electron donor is central to their antimalarial activity, mitochondrial proteins are potential activation sites for the artemisinins. Mutations in the mitochondrial genome, which is 6 kb long and which contains three genes (cytochrome *b*, *COXI*, *COXIII*), could therefore potentially change susceptibility to the artemisinins.

(iv) *ubp-1*, a 3.3-kb gene located on chromosome 2, encodes a deubiquitinating enzyme. Mutations V739F and V770F in *ubp-1* of *P. chabaudi* were recently identified by linkage group analysis of an elegant genetic-cross experiment to confer resistance to artesunate in this rodent malaria parasite (9).

(v) Laboratory-induced artemisinin resistance in the *P. chabaudi* model has been demonstrated in a chloroquine-resistant strain. This suggests that chloroquine resistance in this model might be a prerequisite for the subsequent development of artemisinin resistance. We therefore also assessed the parasite genome for the presence of the *P. falciparum CRT* (*pfCRT*) K76T mutation, which plays a central role in the chloroquine resistance of *P. falciparum*.

We report here the molecular characteristics of these five groups of genes in *P. falciparum* isolates from western Cambodia, where most infections show reduced sensitivity to artesunate, compared to those of strains obtained from northwestern Thailand, where infections are artemisinin sensitive (6).

MATERIALS AND METHODS

Materials. Parasite DNA was collected from patients with falciparum malaria enrolled in clinical studies on artemisinin resistance conducted in Pailin, western Cambodia, and WangPha, northwestern Thailand, in 2007 and 2008 and from studies in Trat Province (eastern Thailand) and MaeSot (northwestern Thailand) in 2006. The clinical trials were registered under ClinicalTrials.gov number NCT00493363 and Current Controlled Trials numbers ISRCTN64835265 and ISRCTN15351875. The results of these studies showed a relatively uniform *in vivo* resistance of *P. falciparum* in the patients studied in western Cambodia in 2007, characterized by an almost doubling of the parasite clearance times compared to those for the patients studied in northwestern Thailand, who had similar plasma concentration profiles for artesunate and dihydroartemisinin (6).

Parasite DNA was extracted from either filter paper (Whatman 3MM) or whole blood using a Qiagen kit (Hilden, Germany).

Quantitation of *pfmdr1* copy number by TaqMan real-time PCR. The *pfmdr1* copy number was assessed by TaqMan real-time PCR on a Corbett Rotor-Gene 3000 apparatus (Corbett Research, Australia). The primers and probes have been described previously (15). A Quantitec multiplex PCR without 6-carboxyl-X-rhodamine (Qiagen) was used, and the temperature profile used was according to the manufacturer's instructions. Amplification was carried out as a multiplex PCR in a total volume of 10 μl. The samples were set up in triplicate. Every amplification run contained nine replicates of the 3D7 control and triplicates without template as negative controls. The β-tubulin gene served as an internal control for the amount of sample DNA added to the reaction mixtures. Normalized relative amounts of the target gene were calculated by using 3D7 as a calibrator. The relative amounts of the target genes were calculated by using the comparative threshold cycle (C_T) method. Copy numbers were calculated using the formula $2^{-\Delta\Delta CT}$, with $\Delta\Delta C_T$ being the difference between the ΔC_T of the unknown sample and the ΔC_T of the reference sample.

Assessment of full lengths of *pfserca*, *pfmdr1*, and mtDNA. Pairs of primers were designed to span 4,068 bp of the *P. falciparum serca* (*pfserca*) gene (9 pairs), 4,260 bp of *pfmdr1* (10 pairs), and 6 kb of the mitochondrial DNA (mtDNA; 14 pairs; Table 1) using the primer 3 program (http://primer3.sourceforge.net/). Each pair of primers was designed to overlap at least 100 bp in order to cover all sequencing results. Different pairs of primers were optimized regarding the

TABLE 1. Sequences of primers used for full-length sequencing and PCR conditions

Sequence GenBank accession no. and gene	Region (bases)	Primer name	Sequence (5′–3′)	Size (bp)	T_m[a] (°C)	Product size (bp)	$MgCl_2$ concn (mM)	Annealing temp (°C)	Remarks
AB121056 (T9/96)									
pfATPase6	6–742	Pfserca_S1F	CTTATTATATCTTTGTCATTCGTG	24	46	737	3	52	
		Pfserca_S1R	CCACATACAATAGCGGTAGATG	22	49				
pfATPase6	555–1367	Pfserca_S2F	AATAAAACTCCCGCTGATGC	20	58	813	2	58	
		Pfserca_S2R	TTCTCCATCATCCGTTAAAGC	21	60				
pfATPase6	994–1837	Pfserca_S3F	TTGCTTTAGCTGTTGCTGCT	20	58	844	1.5	58	
		Pfserca_S3R	TTGTTGATACCCCTTGGTGA	20	58				
pfATPase6	1327–2026	Pfserca_S4F	AAGATGAAGGAAATGTTGAAGC	22	50	700	3	55	
		Pfserca_S4R	CCCAATTTTGAGTGGAAACAA	21	60				
pfATPase6	1940–2489	Pfserca_S5NF	GGCAACAACAAATGGATATGA	21	50	550	2	55	
		Pfserca_S5R	TCCTTTTCATCATCTCCTTCA	21	49				
pfATPase6	2341–3216	Pfserca_S6F	GAGCATTAAGAACACTTAGCTTTGC	25	53	876	1	58	
		Pfserca_S6R	CTGTTGCTGGTAATCCGTCA	20	60				
pfATPase6	2721–3457	Pfserca_S7F	CCAAGAATTGTTTTCTGTAGAACTGA	26	53	737	3	58	
		Pfserca_S7R	CGTGTGCATATCTGAATCTGG	21	62				
pfATPase6	3106–3805	Pfserca_S8F	CTGACGGATTACCAGCAACA	20	60	700	1	60	Difficult sequencing
		Pfserca_S8R	CACTCAAGGCATTCAAAGCA	20	58				
pfATPase6	3526–4068	Pfserca_S9NF	CCAGATTCAGATATGCACACG	24	51	543	1	52	Difficult sequencing
		Pfserca_S9R	ATCAATTTTAATTTTCTTGGTTC	23	56				
M76611									
PfmtDNA[a]	1–546	Pfmt 1-546_F	AAGCTTTTGGTATCTCGTAATG	22	56	546	2	55	
		Pfmt 1-546_R	CAGCTATCCATAGTTAATTGATTCC	25	58				
PfmtDNA	429–959	Pfmt 429-959_F	ATGTGTTCCACCGCTAGTGT	20	59	531	2	55	*COXIII*
		Pfmt 429-959_R	TTGTGTTACAGGATTACATTTTTCTC	26	58				
Pfmt DNA	799–1340	Pfmt 799-1340_F	TTGTAATTTGATCAGTGTGAGG	22	56	542	3	55	*COXIII*
		Pfmt 799-1340_R	TTCTATTCGAGAAAGTTTTTATTCTG	26	57				
Pfmt DNA	1208–1750	Pfmt 1208-1750_F	TGGATATGGTGATAAACTAAAATG	24	56	543	2	55	*COXIII*
		Pfmt 1208-1750_R	ATTACCTTTCCGGCTGTTTC	20	59				
Pfmt DNA	1654–2201	Pfmt 1654-2201_F	CTTGTACACACCGCTCGTC	19	58	548	2	58	*COXI*
		Pfmt 1654-2201_R	TCTTGTGCAATTATTCTTAAAGATG	25	57				
Pfmt DNA	2103–2626	Pfmt 2103-2626_F	TTTATGGTTTTCATTTTTATTTGG	24	57	524	2	58	*COXI*
		Pfmt 2103-2626_R	TGATCAATGACCATGTAGAAACA	23	59				
Pfmt DNA	2482–3025	Pfmt 2482-3025_F	GCTGTAGATGTAATAATTTTTGGTTT	26	57	544	1	55	*COXI*
		Pfmt 2482-3025_R	TCCAGTTAAATACTTTTGTACCG	23	56				

Pfmt DNA	2896–3442	Pfmt 2896-3442_F	GCTGTTTTAGGAAGCTTAGTATGG	24	58	547	1	55	*COXI*
		Pfmt 2896-3442_R	TCATTGTTGACCCAATAGAACA	22	59				
PfmtDNA	3328–3874	Pfmt 3328-3874_F	TTAACATTTTTACCTATGCATTTTT	25	56	547	3	55	*COXI* and *COB*
		Pfmt 3328-3874_R	AAGACATAACCAACGAAAGCA	21	58				
PfmtDNA	3690–4329	Pfmt 3690-4329_F	GAATTATGGAGTGGATGGTG	20	58	640	3	55	*COB*
		Pfmt 3690-4329_R	CAGCTGGTTTACTTGGAA	18	52				
PfmtDNA	4188–4687	Pfmt 4188-4687_F	AGTTTATTTGGAATTATACCTTTATCA	27	56	500	3	55	*COB*
		Pfmt 4188-4687_R	AACCTTACGGTCTGATTTGTTC	22	58				
PfmtDNA	4596–5140	Pfmt 4596-5140_F	GATTACAGCTCCCAAGCAAA	20	58	545	1	55	
		Pfmt 4596-5140_R	AGACCGAACCTTGGACTCTT	20	58				
Pfmt DNA	4965–5573	Pfmt 4965-5573_F	GTTCGGTATTGCATGCCTG	19	58	609	1	55	
		Pfmt 4965-5573_R	CTTATGTGTTGGCATGGTT	19	54				
Pfmt DNA	5353–5945	Pfmt 5353-5945_F	GAAATCCGTATATCGATGTC	20	56	593	1	55	
		Pfmt 5353-5945_R	GTTGAACATAGGCTGAGTC	19	56				
XM 001351751									
Pfmdr1	1–504	Pfmdr1_1-504_F	CATTTTATTTGATTTTGTGTTGAAA	25	50	504	3	57	
		Pfmdr1_1-504_R	CGTACCAATTCCTGAACTCAC	21	49				
Pfmdr1	329–878	Pfmdr1_329-878_F	TGATATCAAGTTATTGTATGGATGTAA	27	49	550	3	57	
		Pfmdr1_329-878_R	CCGAATGCATAAGAAACTAAAA	22	49				
Pfmdr1	728–1261	Pfmdr1_728-1261_F	CTGTTGCAAGTTATTGTGGAGA	22	50	534	3	57	
		Pfmdr1_728-1261_R	TTGATTTCCCACAACCTGAT	20	56				
Pfmdr1	1155–1684	Pfmdr1_1155-1684_F	TCATTATGATACTAGAAAAGATGTTGA	27	49	530	3	57	
		Pfmdr1_1155-1684_R	TGGATGCATTGGAACCTACT	20	58				
Pfmdr1	1589–2131	Pfmdr1_1589-2131_F	CTGATGTTGTTGATGTGTCCA	21	49	543	3	60	
		Pfmdr1_1589-2131_R	CCGATCCATTATCATTTCCA	20	56				
Pfmdr1	2013–2539	Pfmdr1_2013-2539_F	TGAACAAGGTACACATGATAGTCTT	25	49	527	3	57	
		Pfmdr1_2013-2539_R	TGTTTCTGAAATGAACATAGCAA	23	50				
Pfmdr1	2401–2911	Pfmdr1_2401-2911_F	GGATTATATCCCGTATTTGCTTT	23	51	511	3	57	
		Pfmdr1_2401-2911_R	CAAAAACTCCGCTTGACATA	20	56				
Pfmdr1	2780–3279	Pfmdr1_2780-3279_F	ATTTTTGTCCAATTGTTGCAG	21	50	500	3	57	
		Pfmdr1_2780-3279_R	TGATAATTTTGCATTTTCTGAATC	24	50				
Pfmdr1	3179–3777	Pfmdr1_3179-3777_F	TTGATGACTTTATGAAATCCTTATT	25	49	599	3	57	
		Pfmdr1_3179-3777_R	CATGGGTTCTTGACTAACTATTGA	24	50				
Pfmdr1	3639–4260	Pfmdr1_3639-4260_F	TCAAACCAATCTGGATCTGC	20	58	622	3	57	
		Pfmdr1_3639-4260_R	GCTTCATTTAGCTAATTTTACATATTTTT	29	52				

[a] PfmtDNA, *P. falciparum* mtDNA.

reaction conditions and the temperature profile. Single PCR amplifications were performed in a 100-μl total sample volume. The DNA sequences were assessed by direct sequencing of the PCR product (Macrogen, South Korea) and were analyzed with the Bioedit bioinformatics program.

Assessment of point mutations in *pfcrt* and *ubp-1*. The *pfcrt* gene was amplified from the DNA template using nested PCR. A PCR-restriction fragment length polymorphism assay was then used to assess the mutation at position 76 using ApoI (New England Biolabs, United Kingdom), as described previously (7). Digestion fragments were analyzed on a 3% agarose gel. The *ubp-1* gene encoding a deubiquitinating enzyme was amplified from the DNA template using nested PCR, as described previously (9). PCR products were sent for DNA sequencing (Macrogen), and the Bioedit software program was used for the analysis.

Statistical analysis. Statistical analysis was done with the STATA (version 10) and Epistat programs. Since the current definition of artemisinin resistance is based solely on the parasite clearance rates in patients (6), which also partly depend on host factors, we do not know whether the parasite strains from the small subset of patients in Pailin in western Cambodia with relatively fast clearance rates consist of a separate, distinct sensitive population or merely represent a genetically resistant population at the faster end of a spectrum of resistance development. Therefore, the analysis compares the genetic differences in parasites between the two geographical areas, as well as between parasites obtained from patients with fast versus slow clearance rates. Comparisons between sites were conducted for each genotype using the chi-square test or Fisher's exact test, as appropriate.

To compare the fast and slow responders, the clearance rate was calculated for each patient. This was defined as the slope of the best-fitting line when the $\log_{10}$ parasitemia was plotted against the time since the start of treatment. Using the 90% cutoff reported by Anderson et al. (3), patients with a clearance rate of 0.065 or less were classified as slow responders. Comparisons were made using Fisher's exact test and were adjusted for site.

RESULTS

Candidate genes for artemisinin resistance were compared according to study site and parasite clearance rates (Table 2).

***pfmdr1* copy number and mutations.** Parasite DNA obtained from patients in Pailin (2005 and 2007), WangPha, and Trat was assessed for *pfmdr1* copy number variations using real-time PCR. *pfmdr1* amplification was found in 50% of all samples from WangPha (Thailand), and the median copy number was 1.47 (range, 0.75 to 3.35 copies). Multiple copies were present in 45.9% of the samples from Trat Province in eastern Thailand, close to Cambodia (median = 2.28; range = 0.87 to 3.53), and in 33% of samples from Pailin collected in 2005 (median = 1.23; range = 0.85 to 2.65). In contrast, parasite DNA from patients in Pailin in 2007 showed multiple *pfmdr1* copies in only 2 of 43 primary infections (5%), with both having a copy number of 2 ($P < 10^{-6}$; Table 2).

Full-length sequencing of *pfmdr1* was performed for samples from Pailin and WangPha collected in 2007. Mutations at positions N86Y, S1034C, and D1246Y were not detected in any of the samples; but mutant codon Y184F was present in 32 (78%) of the parasites from Pailin and in 2 (5%) from WangPha ($P < 10^{-8}$). Six novel nonsynonymous mutations at E130K, L327H, D587E, S784L, and F1226Y, one synonymous mutation at a747g, and an indel at position 661N were found (Fig. 1). The mutations at L327H, D587E, S784L, and N1042D were observed only at a low frequency in Pailin. The other two mutations, E130K and F1226Y, were found only in WangPha ($P < 10^{-8}$). The indel of N at position 661N (using 3D7 [GenBank accession number XM_001351751] as a reference) was found in both areas (Table 2).

Sequencing of full-length *pfserca*. Sequencing of the full length of the *pfserca* gene, spanning 4,049 bp with 3 exons and 2 introns, revealed only sporadic point mutations compared to the wild-type sequence. Mutations at codons L263E and S769N, which have been proposed to confer artemisinin resistance, were not detected. Mutations occurred only sporadically in both areas and were situated at positions I89T, H243Y, A438D, t2951a, N465S, and E847K, as shown in Table 2. Indels of TA located in intron nucleotides 3621 to 3671 (T9/96 [GenBank accession number AB121056] was used as a reference) were observed at both sites, had repeats that varied from 10 to 19, and were similar in length in both areas.

Mutations in *pfcrt* and *ubp-1*. The K76T mutation in the *pfcrt* gene was present in all parasites from both Pailin and Wang-

TABLE 2. Mutations of *pfmdr1*, *pfATPase6*, 6-kb mitochondrial genome, *ubp-1*, and *pfCRT* in samples from Pailin and WangPha[a]

Candidate gene	No. of corresponding genotypes (total no. of patients)		*P* value
	Pailin (2007)	WangPha	
pfmdr1 (multidrug resistance)			
Single copy no.	41 (43)	19 (35)	
Multiple copy no.	2 (43)	16 (35)	$<10^{-6}$
E130K mutant	0 (40)	6 (27)	0.003
Y184F mutant	32 (41)	2 (37)	$<10^{-8}$
L327H mutant	5 (43)	ND	
D587E mutant	1 (40)	0 (30)	1.000
S784L mutant	12 (43)	0 (20)	0.006
N1042D mutant	1 (42)	0 (39)	1.000
F1226Y mutant	0 (43)	18 (33)	$<10^{-8}$
pfmdr1 (indels)			
Wild type	2 (41)	0 (29)	0.333
Insertion 1 amino acid + N	20 (41)	17 (29)	0.222
Insertion 3 amino acids + NNN	6 (41)	0 (29)	0.030
Deletion 1 amino acid − N	0 (41)	8 (29)	0.168
Deletion 3 amino acids − NNN	13 (41)	4 (29)	0.460
pfATPase6 (SERCA)			
I89T mutant	8 (43)	2(96)[b]	0.118
H243Y mutant	0 (43)	1 (38)[b]	0.480
L263E mutant	0 (43)	1 (96)[b]	0.480
A438D mutant	1 (40)	2 (56)[b]	0.490
N464 deletion	1 (43)	0 (56)[b]	0.510
N465S mutant	5 (42)	1 (56)[b]	0.118
N465deletion	1 (43)	0 (56)[b]	0.518
E847K mutant	2 (39)	0 (86)[b]	0.253
PfDUB (deubiquitinating enzyme)			
V739F wild type	17 (17)	20 (20)	0.999
V770F wild type	17 (17)	20 (20)	0.999
pfcrt (chloroquine resistance transporter), K76T mutant	43 (43)	37 (37)	0.999
P. falciparum mtDNA (mtDNA genome)			
I239V mutant (*COXIII*)	19 (38)	23 (49)[b]	0.473
A312V mutant (cytochrome *b*)	0 (42)	1 (45)[b]	0.523

[a] Mutation frequencies were determined by full-length sequencing and copy number polymorphism for *pfmdr1*, full-length sequencing for *pfATPase6*, and full-length sequencing for the 6-kb mitochondrial genome and by the detection of specific mutations for *ubp-1* (which encodes a deubiquitinating enzyme) and *pfCRT*.

[b] The results were obtained for *P. falciparum* parasites collected in 2006 in MaeSot, Thailand. No mutations were found at position N86Y, S1034C, or D1246Y in the *pfmdr1* gene.

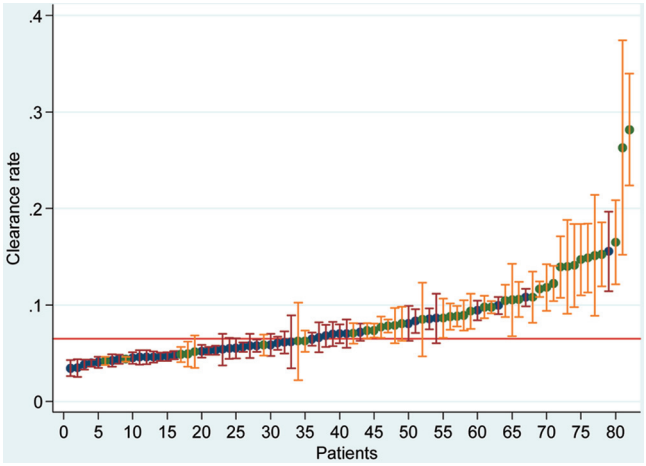

FIG. 2. Distribution of clearance rates in patients from Pailin (blue dots) and Maesot (green dots). Clearance rates of less than 0.065 (red line) were considered low. Bars, 95% confidence intervals.

Pha. The *ubp-1* gene encoding a deubiquinating enzyme was assessed for the V739F and V770F mutations, which are the equivalent of the mutations conferring artemisinin resistance in the rodent model of malaria (9), but no mutations were detected.

Sequencing of full-length mtDNA. The whole mitochondrial genome of 6 kb in length was assessed using directed PCR sequencing with 14 overlapping paired primers. In the coding region, only one nonsynonymous mutation was observed in a coding region, which was the I239V mutation in the *COXIII* gene, present in 50% (19/38) of the parasites (Table 2). The frequency of this mutation did not differ between study sites. Four mutations were found in intergenic regions, which were located at nucleotides a74t, c204t, t1776c, and t2686c, all with relatively low frequencies.

Comparison according to parasite clearance rates. There were 28/42 patients (66.7%) in western Cambodia and 8/40 (20%) in northwestern Thailand with a slow parasite clearance rate (Fig. 2), defined as a slope of the log parasite-time curve of less than 0.065 (3). Differences in the frequencies of mutations between the fast and slow responders were observed only for *pfmdr1* F1226Y ($P = 0.03$) and Y184F ($P = 0.001$). For F1226Y, there was a lower frequency of mutations in the slow responders (4/35 versus 14/40 for the fast responders), while for Y184F, there was a higher frequency of mutations among slow responders (22/33 versus 12/44 for the fast responders). The association for Y184F did not persist when the results were adjusted for site ($P = 0.54$). The results for F1226Y were not adjusted for site, as there were no mutations in any of the Pailin samples (Table 2). The frequencies of none of the other genes showed significant differences between sites.

DISCUSSION

This study investigated genes which have been proposed to be potential contributors to artemisinin resistance: *pfmdr1*, *pfserca*, *pfcrt*, *ubp-1*, and mtDNA. The only mutation that was more frequent in parasites with slow parasite clearance rates was Y184F in *pfmdr1*. However, if the analysis was adjusted for both study site and clearance rate, the association was no longer significant, suggesting that it is unlikely that the Y184F mutation plays a causal role in reduced artemisinin resistance.

pfmdr1 gene amplification was not associated with reduced artemisinin susceptibility. In fact, the opposite was found. In western Cambodia, the *pfmdr1* copy numbers were significantly lower in the parasite samples obtained in 2007 than in those obtained 2 years previously, in 2005. This coincided with a change in drug policy in the region from the use of artesunate-mefloquine to the use of dihydroartemisinin-piperaquine in 2006. This has considerably diminished the mefloquine drug pressure in the Pailin area. *In vitro*, *pfmdr1* amplification can readily be induced by exposure to low concentrations of mefloquine, and it has been estimated that the frequency of amplification and deamplification of the gene is very high, at about one event every 10^8 mitoses during the asexual cycle of *P. falciparum* (14). This high frequency of amplification and deamplification fits with the observations made in the current study, with rapid deamplification of *pfmdr1* copy numbers occurring after mefloquine pressure is removed from the population. In the absence of mefloquine, the amplified *pfmdr1* gene and gene products confer a fitness disadvantage (14), possibly through the increased energy demands of the ATP-consuming ATP-binding cassette transporter.

PfATPase6, the product of the *pfserca* gene, has been suggested to be a specific target of the artemisinin drugs (8). *In vitro* resistance to artemether in parasites obtained from French Guiana (but not Cambodia) was linked to the S769N mutation in *pfserca*. In the present study, mutations in the *pfserca* gene were infrequent in both western Cambodia and northwestern Thailand, and there was no clear pattern, excluding mutations in this gene as a cause of artemisinin resistance.

The mitochondrial inner membrane is a site of intense electron transfer, which is inherent to its function, and is thus a potential site of artemisinin activation, which requires reduction of the unique endoperoxide bridge generating carbon-based free radicals (12). Although mutations in the *COXIII* gene at the I239V position were found in 50% of the parasites, the frequency of this mutation did not differ between sites with artesunate-resistant and -sensitive parasite strains.

The *ubp-1* gene encoding a deubiquitinating enzyme was identified as the gene conferring artemisinin resistance in a rodent model of infection with *P. chabaudi*. The equivalent mutations, V739F and V770F, however, were not identified in *P. falciparum.* Underlying differences in chloroquine resistance do not confound the lack of correlation between the candidate genes and artemisinin resistance, since the *pfCRT* K76T mutation was present in all parasite strains.

Microsatellite typing strongly suggests that a slow parasite clearance rate in patients with uncomplicated falciparum malaria in Pailin is a heritable trait of the parasite (3), implying that a genetic change in the parasite confers artemisinin resistance rather than epigenetic causes. Since mutations (and for *pfmdr1* also amplification) in the candidate genes investigated here do not account for artesunate resistance, other approaches have to be followed. These include whole-genome sequencing of resistant parasite genes for comparison with the sequences of sensitive strains, analysis of gene expression profiles using microarrays, microsatellite mapping, and genome-wide hybridization and single nucleotide polymorphism map-

ping (2, 13, 15, 16). These findings do not exclude the possibility that the gene products of the genes examined play a role in the mechanism of artemisinin's action, since resistance can be conferred through a variety of mechanisms, such as changes in influx and efflux mechanisms. Also, in case artemisinin resistance is conferred by multiple gene mutations, with each contributing to a different extent to the resistant phenotype, the small sample size of this study might have precluded their detection.

In summary, although artemisinin resistance in western Cambodia seems to be a heritable genetic trait, none of the candidate genes suggested by earlier studies confer artemisinin resistance. The use of a genome-wide approach by whole-genome sequencing and gene expression transcriptome studies to identify the molecular basis of artemisinin resistance is now indicated.

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